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Parasite sequestration in *Plasmodium falciparum* malaria: Spleen and antibody modulation of cytoadherence of infected erythrocytes

(monkey/endothelium/surface antigen/cell binding)

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ABSTRACT **Sequestration, the adherence of infected erythrocytes containing late developmental stages of the parasite (trophozoites and schizonts) to the endothelium of capillaries and venules, is characteristic of *Plasmodium falciparum* infections. We have studied two host factors, the spleen and antibody, that influence sequestration of *P. falciparum* in the squirrel monkey. Sequestration of trophozoite/schizont-infected erythrocytes that occurs in intact animals is reduced in splenectomized animals; *in vitro*, when infected blood is incubated with monolayers of human melanoma cells, trophozoite/schizont-infected erythrocytes from intact animals but not from splenectomized animals bind to the melanoma cells. The switch in cytoadherence characteristics of the infected erythrocytes from nonbinding to binding occurs with a cloned parasite. Immune serum can inhibit and reverse *in vitro* binding to melanoma cells of infected erythrocytes from intact animals. Similarly, antibody can reverse *in vivo* sequestration as shown by the appearance of trophozoite/schizont-infected erythrocytes in the peripheral blood of an intact animal after inoculation with immune serum. These results indicate that the spleen modulates the expression of parasite alterations of the infected erythrocyte membrane responsible for sequestration and suggest that the prevention and reversal of sequestration could be one of the effector mechanisms involved in antibody-mediated protection against *P. falciparum* malaria.**

Among the four species of malaria infecting man, one distinguishing feature of *Plasmodium falciparum* is that only erythrocytes containing young forms of the parasite (rings) are found in the peripheral blood (1); erythrocytes containing more mature forms (trophozoites and schizonts) are sequestered in the postcapillary venules of various organs where they adhere to endothelial cells (2). The mechanisms of parasite sequestration remain unclear. Ultrastructural studies have suggested that adherence of parasitized erythrocytes to the vascular endothelium occurs by means of excrescences on the infected erythrocyte membrane that have been called knobs (3, 4); antigens present on these knobs are recognized by immune serum of the host (5, 6). Cytoadherence of infected erythrocytes can be studied *in vitro* through the binding of infected erythrocytes to monolayers of human endothelial cells (7); infected erythrocytes also bind to monolayers of human melanoma cells, which can replace endothelial cells for *in vitro* studies of parasite sequestration (8).

Sequestration favors the development of the parasite by protecting it from the filtering action of the spleen (9); it is also responsible for the severe clinical forms of cerebral malaria in which brain capillaries are obstructed by sequestered parasites. Means of inhibiting sequestration may therefore hinder parasite development and alleviate the clinical severity of the disease.

In the *P. falciparum* infection of the squirrel monkey, we have studied two host factors that influence both sequestration *in vivo* and adherence to melanoma cells *in vitro*—namely, the spleen and antibody. First, by comparing the cytoadherence properties of infected erythrocytes in nonsplenectomized and in splenectomized monkeys infected with the same strain of the parasite, we have shown that, as a result of splenectomy, sequestration *in vivo* is reduced and that infected erythrocytes from these splenectomized animals no longer bind to melanoma cells *in vitro*. Second, the passive transfer of immune serum into an infected intact animal can reverse sequestration; this correlates with the fact that antibody can inhibit and reverse binding of infected erythrocytes to melanoma cells *in vitro*.

MATERIALS AND METHODS

Animals. Female Bolivian squirrel monkeys (*Saimiri sciureus*) (South American Primates, Miami, FL) were used throughout this study. Nonsplenectomized and splenectomized animals will be referred to as S^+ and S^- animals, respectively.

Parasites. The Ugandan Palo Alto/PLF-3 strain of *P. falciparum* adapted to the squirrel monkey (10) and the derived clone, PLF-3/B11 (11), were used. In view of the differences observed in ref. 11 between parasites maintained in S^+ and S^- animals, we maintained two different lines by serial transfer in either S^+ or S^- animals.

Animals were infected by intravenous injection of infected blood. Parasites were obtained by venipuncture of anesthetized infected animals. Infected blood was cryopreserved and thawed according to the method described of Diggs *et al.* (12). Short-term culture of fresh or thawed infected blood was carried out as described by Trager and Jensen (13) except that fetal bovine serum was substituted for human serum.

Immune Sera. Immune serum $31/S^+$ was obtained in the following manner: A S^+ animal was infected with parasites from a S^+ animal; after self-cure of this infection, the animal was challenged three times by intravenous inoculation of infected blood from a S^+ animal; none of these challenges were followed by detectable parasitemia. Serum was collected 7 days after the last challenge. Immune serum $30/S^-$ was obtained in a similar

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Abbreviations: S^+, nonsplenectomized; S^-, splenectomized; MCBA, melanoma cell-binding assay; TC medium, tissue culture medium.

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fashion from a S^- animal infected and challenged three times with parasites from a S^- animal.

Tissue Culture Medium. The tissue culture (TC) medium used for both the culture of melanoma cells and malaria parasites was RPMI 1640 medium containing glutamine and sodium bicarbonate (M A Bioproducts, Walkersville, MD), 25 mM Hepes, gentamicin at 0.1 mg/ml, and 10% heat-inactivated fetal bovine serum (Flow Laboratories).

Melanoma Cells. Amelanotic human melanoma cells (American Type Culture Collection no. CRL 1585, designation C32r) were put into culture as in ref. 8, with the following modifications. Cells were suspended in TC medium at a concentration of 2×10^5/ml; 0.5 ml of this cell suspension was layered on a 2.2-mm glass coverslip in a 35-mm plastic Petri dish. Cells were allowed to settle and stick to the coverslip overnight at 37°C in humidified 5% CO_2/95% air; 1.5 ml of TC medium was then added. Binding assays were carried out 24–48 hr after plating of the melanoma cells.

Melanoma Cell-Binding Assay (MCBA). When *P. falciparum*-infected blood is incubated with monolayers of human melanoma cells, trophozoite/ and schizont-infected erythrocytes specifically bind to these cells; there is no binding of uninfected erythrocytes or of erythrocytes infected with rings (8). To more readily quantify the results of this type of MCBA, we labeled the erythrocytes with ^{51}Cr and modified the assay described in ref. 8 in the following manner: Infected blood was cultured for 24 hr to allow partial maturation of the ring stages. Parasitemia was adjusted to 2–5% by addition of normal *Saimiri* erythrocytes; cultures were then centrifuged and the pellet was suspended to a 5% cell suspension in TC medium. One millicurie of sodium chromate (New England Nuclear; 1 mCi/ml in sterile saline; 1 Ci = 37 GBq) was added to 4 ml of cell suspension. After 1 hr at 37°C in humidified 5% CO_2/95% air, the labeled erythrocytes were washed three times with TC medium and the pellet was suspended to a 20% suspension in TC medium. Medium was aspirated from the melanoma cell-containing Petri dishes and replaced with 1 ml of fresh TC medium to which 100 μl of the labeled erythrocyte suspension was added. Dishes were incubated at 37°C in 5% CO_2/95% air for 1 hr on a rocking platform (25 cycles per min); coverslips were then removed, washed by gentle dipping in five successive baths of TC medium, and put into fresh Petri dishes containing 1 ml of TC medium supplemented with 100 μl of 5% Nonidet P-40; the medium containing the ^{51}Cr freed from the lysed erythrocytes bound to the melanoma cells was transferred into counting vials and radioactivity was measured with a Packard 5230 gamma counter.

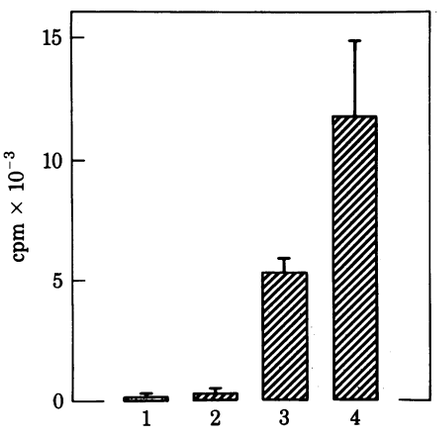

FIG. 1. Effect of parasite development on binding of infected erythrocytes to melanoma cells *in vitro*. Bars: 1, normal (uninfected) erythrocytes; 2–4, parasitized blood from a S^+ animal (6% parasitemia, synchronous infection) cultured for 6, 24, and 36 hr, respectively. The 6-hr sample was 100% rings, the 24-hr sample was 20% rings/77% trophozoites/3% schizonts, and the 36-hr sample was 4% rings/10% trophozoites/86% schizonts. Results are expressed as mean ± 2 SD release of ^{51}Cr from detergent-lysed erythrocytes bound to melanoma cells in duplicate cultures.

As shown in Fig. 1, binding measured in the MCBA correlated with parasite maturation; ring-infected erythrocytes did not bind to the melanoma cells, binding appeared at the trophozoite stage, and increased with parasite maturation from the trophozoite stage (6,000 cpm) to the schizont stage (12,000 cpm).

Treatment of Erythrocytes with Enzymes. Erythrocytes (5×10^8; 4% parasitemia) were incubated in 1 ml of TC medium without serum containing different concentrations of trypsin (Sigma), chymotrypsin (Worthington), or neuraminidase (GIBCO) for 30 min at 37°C. Cells were washed three times with 10 ml of TC medium. Trypsin-treated cells were then incubated for 15 min at 37°C with soybean trypsin inhibitor (Millipore; 1 mg/ml), and chymotrypsin-treated cells were incubated with phenylmethylsulfonyl fluoride (Sigma; 2 mM); cells were then washed three times with TC medium. After enzyme treatment, erythrocytes were either labeled and used in the MCBA or cultured for 24 hr before labeling and MCBA.

Electron Microscopy. Erythrocytes infected with clone B-11 taken from a S^- or a S^+ squirrel monkey were cultivated *in vitro* for 28 hr to obtain an optimal level of schizont-infected cells. The cell suspension was fixed for 15 min on ice in an equal volume of Karnovsky's aldehyde fixative and further processed as described (14). Stained thin sections were examined in a JEOL 100C electron microscope.

RESULTS

Differences in the Degree of *in Vivo* Parasite Sequestration in S^+ and S^- Animals as Reflected by Peripheral Blood Smears. The differences between the evolution of parasitemia in a S^+ and a S^- animal are shown in Fig. 2. Daily blood smears from the S^+ animal showed that mainly ring forms were present in the peripheral blood, reflecting a high level of parasite sequestration; the parasitemia evolved in a sawtooth manner, de-

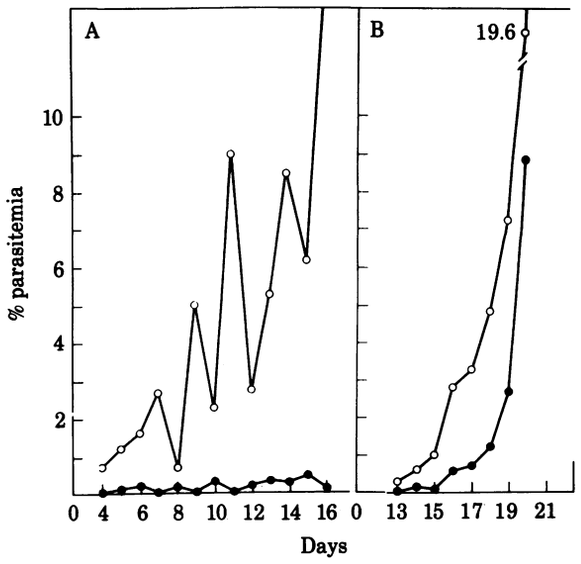

FIG. 2. Evolution of parasitemia in S^+ (*A*) and S^- (*B*) monkeys. S^+ and S^- monkeys were inoculated intravenously with a stabilized form of infected blood (S^+ and S^- line, respectively). Thin blood films were made daily and the percentage of erythrocytes containing rings (○) or trophozoite/schizonts (●) was determined.

creasing every other day when the majority of parasites were at the trophozoite/schizont stage and were thus sequestered in the tissues. This contrasted with what could be seen in a S^- animal in which, at any one time, a mixture of different developmental stages could be found in the peripheral blood, indicating that parasite sequestration was absent or dramatically reduced; parasitemia increased in a continuous fashion and consisted of a mixture of both ring and trophozoite/schizont stages. Such patterns were consistently observed in other animals, although the length of the prepatent period varied greatly from one animal to another.

Comparison of Infected Erythrocytes from S^+ and S^- Animals in the MCBA. Was the decreased sequestration observed in S^- animals merely a consequence of the removal of a potential splenic reservoir for trophozoite/schizont-infected erythrocytes or was it related to an alteration of the cytoadherence properties of the infected erythrocyte? We compared the *in vitro* binding to melanoma cells of infected erythrocytes from S^+ and S^- monkeys by using infected blood samples with a similar parasitemia and degree of maturation. As shown in Fig. 3, only infected erythrocytes from S^+ hosts bound to melanoma cells. The absence of binding of infected erythrocytes from S^- animals was confirmed in 11 experiments involving four different S^- monkeys.

Evolution of *in Vitro* Binding Characteristics of the Cloned Parasite PLF-3/B11 After Transfer from a S^- into a S^+ Monkey. Cloned PLF-3/B11 was used to explore if and when a cloned parasite could switch the *in vitro* cytoadherence properties of infected erythrocytes from nonbinding to binding. Blood from a S^- monkey infected with clone PLF-3/B11 was inoculated into a S^+ monkey. Infected erythrocytes from the inoculum did not bind. The MCBA was carried out 8 and 20 days after inoculation. By day 8 after inoculation, the parasites had gone through four cycles of multiplication and the majority of parasites had invaded the erythrocytes of the S^+ host. At this time, there was no binding of infected erythrocytes in the MCBA. In contrast, infected erythrocytes did bind in the MCBA on day 20 and binding ability was maintained after inoculation into another S^+ animal. Values obtained for binding of the cloned parasite in the S^+ and S^- monkeys are shown in Fig. 4.

Ultrastructure of the Infected Erythrocyte Surface. Comparison of clone B-11 in erythrocytes of S^+ and S^- monkeys showed that both populations had knobs on their surface, confirming earlier studies carried out with the parent line (14).

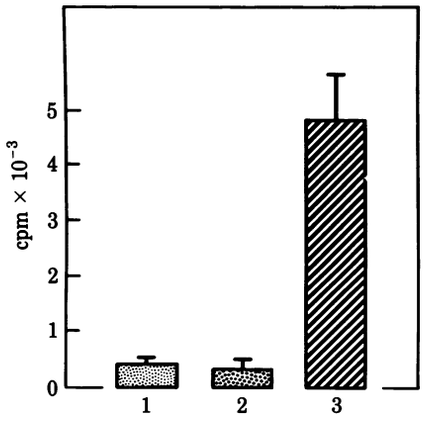

FIG. 3. Effect of splenectomy on binding of infected erythrocytes to melanoma cells. Bars: 1, normal erythrocytes; 2, infected blood from a S^- animal (4.5% parasitemia/>80% trophozoite/schizonts); 3, infected blood from a S^+ animal (5.2% parasitemia/>80% trophozoite/schizonts).

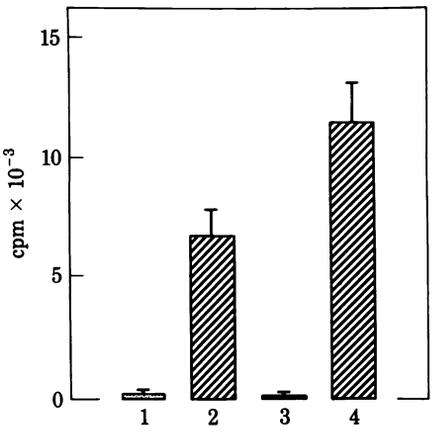

FIG. 4. Binding of cloned and uncloned parasites. Bars: 1, normal erythrocytes; 2, PLF-3/S^+ (original uncloned parasite population in an intact animal; 6% parasitemia/>80% trophozoite/schizonts); 3, B-11/S^- (cloned parasite population from a splenectomized animal; 4.1% parasitemia/>80% trophozoite/schizonts); 3, B-11/S^+ (cloned parasite population in an intact animal after switch, 5.5% parasitemia/>80% trophozoite/schizonts).

Effect of Enzyme Treatment of Infected Blood on Results of the MCBA. After 24 hr in culture, when parasites from S^+ animals had developed from rings to mature trophozoites or schizonts, infected blood was treated with neuraminidase, chymotrypsin, or trypsin. Treatment with trypsin at 1 μg/ml or chymotrypsin at 10 μg/ml totally abolished binding to melanoma cells. In contrast, treatment with neuraminidase at 25 units/ml increased binding of infected cells of the S^+ line by 50%; it did not affect the lack of binding of normal erythrocytes, of erythrocytes infected with ring-stage parasites, or of erythrocytes infected with schizonts from S^- animals. To gain additional information on the origin of the alterations of the infected erythrocyte membrane responsible for the binding, infected blood was treated with trypsin, washed, and then cultured for an additional 24 hr to allow parasite maturation. When carried out at this early stage, enzyme treatment did not inhibit binding to melanoma cells of the treated cells 24 hr later.

Inhibition and Reversal of *in Vitro* Binding by Immune Serum. The binding assay of infected erythrocytes from S^+ animals was carried out in the presence of various dilutions of serum 31/S^+ and serum 30/S^-. As shown in Fig. 5, serum 31/S^+ totally inhibited binding up to a dilution of 1:100; the in-

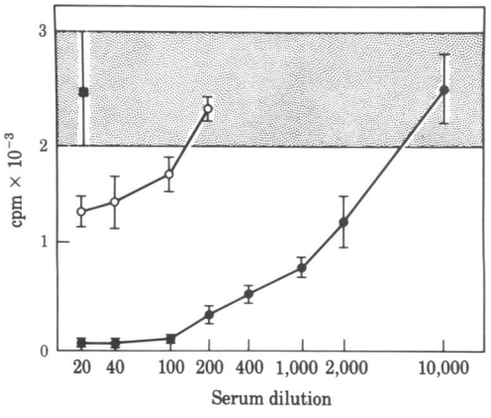

FIG. 5. Inhibition by immune serum of binding of infected erythrocytes to melanoma cells. Binding of infected erythrocytes from a S^+ animal (3% parasitemia/>80% trophozoite/schizonts) was carried out in the presence of increasing dilutions of serum 31/S^+ (●) and serum 30/S^- (○). ▨, Binding ± 2 SD in the presence of normal serum.

hibitory effect was significant up to a dilution of 1:2,000. With serum 30/S$^-$, no more than 50% inhibition could be obtained and binding was no longer inhibited at a dilution higher than 1:100.

We also studied the ability of serum 31/S$^+$ to reverse binding. Binding of infected erythrocytes to melanoma cells was allowed to occur in the absence of immune serum; coverslips coated with melanoma cells to which infected erythrocytes had bound were then rinsed and incubated for 1 hr at 37°C with a 1:20 dilution of serum 31/S$^+$ or normal serum. The results of three experiments are given in Table 1. Up to 99% of the bound erythrocytes were detached by immune serum while only 22% were detached in the controls. Inhibition and reversal of binding were confirmed by using two other immune sera from different S$^+$ animals.

Reversal of *in Vivo* Parasite Sequestration by Inoculation of Immune Serum. Two S$^+$ animals were infected with parasites from a S$^+$ animal. When parasitemia reached 5 and 13%, respectively, each monkey received an intravenous injection of 1.5 ml of serum 31/S$^+$; parasitemia was monitored before and immediately after serum transfer (times 0, 5, 15, 30, 60, and 120 min). Before inoculation with immune serum, few trophozoite/ or schizont-infected erythrocytes were seen in the peripheral blood, reflecting the parasite sequestration that occurs in S$^+$ animals. The number of trophozoite/schizonts in the peripheral bloodstream increased sharply during the first half hour after serum injection and then slowly decreased (Fig. 6). Twenty-four hours after serum transfer, parasitemia was drastically reduced (from 5.0 to 0.3% and from 13.0 to 0.4%) and more than 40% of the parasites present consisted of abnormal trophozoites and schizonts ("crisis forms"). This was followed by a decrease of the parasitemia to less than the detectable level. Neither the immediate effect on circulating trophozoites and schizonts nor the delayed effect on total parasitemia were observed in animals injected with normal serum.

DISCUSSION

We have examined the effects of splenectomy and of antibody on cytoadherence of infected erythrocytes and have begun to define the nature of the alterations of the infected erythrocyte surface involved in sequestration.

We have shown that *P. falciparum*-infected erythrocytes from S$^+$ and S$^-$ squirrel monkeys differ in their ability to be sequestered *in vivo* and to bind to melanoma cells *in vitro*. Infected erythrocytes from S$^+$ animals bind massively to melanoma cells, in contrast to infected erythrocytes from S$^-$ animals, which do not bind at all. This correlates with the decreased parasite sequestration observed in S$^-$ animals. Such differences in cytoadherence properties of infected erythrocytes from S$^+$ and S$^-$ squirrel monkeys show that the spleen plays a major role in modulating the erythrocyte surface alterations responsible for parasite sequestration.

Table 1. Reversal of *in vitro* binding of infected erythrocytes to melanoma cells by immune serum 31/S$^+$

	Exp. 1	Exp. 2	Exp. 3	Mean ± 2 SD
% detached with normal serum	20	16	30	22 ± 7.0
% detached with serum 31/S$^+$	97	100	99	98 ± 1.5

The MCBA was carried out in the absence of immune serum. After washing, melanoma cells covered with infected erythrocytes were incubated for 1 hr with a 1:20 dilution of serum 31/S$^+$ or of normal serum. ^{51}Cr in the culture supernatant was then measured and related to ^{51}Cr bound to the melanoma cells before incubation with serum.

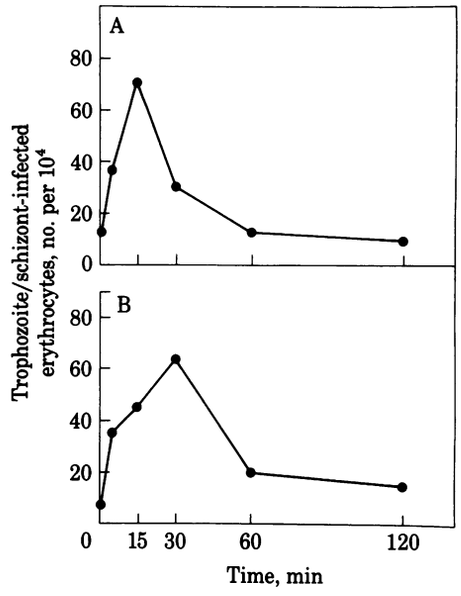

FIG. 6. Kinetics of release of trophozoite/schizont-infected erythrocytes in the peripheral bloodstream after passive transfer of immune serum; 1.5 ml of serum 31/S$^+$ was inoculated at time 0 into two S$^+$ animals, A and B. The number of trophozoite/schizont-infected erythrocytes was counted in the peripheral blood.

The mechanisms by which the spleen could influence the cytoadherence properties of infected erythrocytes include (*i*) initial differences in erythrocytes between uninfected S$^+$ and S$^-$ animals, (*ii*) direct modification of the surface of infected erythrocytes by the spleen, (*iii*) selection of nonbinding mutants, and (*iv*) induction of changes in expression in a cloned parasite population. Results obtained with clone PLF-3/B11 show that, 8 days after the infection of a S$^+$ animal with parasites from a S$^-$ animal, erythrocytes from the S$^+$ host containing trophozoites or schizonts still do not bind *in vitro*. This excludes the possibility that differences in cytoadherence in S$^+$ and in S$^-$ animals are related to initial differences in the erythrocyte populations from the S$^+$ and S$^-$ monkeys; it also argues against the hypothesis that the spleen secondarily modifies the parasite-induced alterations of the infected erythrocyte membrane responsible for sequestration. By day 20, infected erythrocytes bind *in vitro*; this switch in cytoadherence properties suggests that the spleen induces a change in parasite expression on the surface of the infected erythrocyte. This is comparable with what has been observed with surface antigens on *Plasmodium knowlesi*-infected erythrocytes; a parasite clone can lose the ability to express variant-specific erythrocyte surface antigens after passage into a splenectomized rhesus monkey (15).

Few observations have been reported concerning the evolution of *P. falciparum* in splenectomized human hosts. Splenectomy performed in a patient infected with *P. falciparum* has been reported to have modified the pattern of parasitemia during a subsequent attack of malaria (16). In this patient, a high level of parasitemia contrasted with the absence of any severe clinical symptoms related to sequestration and peripheral blood was shown to contain not only the ring forms but all different stages of parasitic development. These peculiar characteristics of the infection suggest that, as in splenectomized monkeys, parasite sequestration was reduced in this splenectomized patient.

Knobs have been shown to mediate the binding of trophozoite/ and schizont-infected erythrocytes to the vascular endothelium (3, 4). Although infected erythrocytes from S$^-$ animals fail to bind *in vitro* and to be sequestered *in vivo*, both

infected erythrocytes from S^+ and S^- monkeys have knobs that are indistinguishable in morphology and numbers. This indicates that an ultrastructural description is not sufficient to define a functional binding site. *P. falciparum* in S^- monkeys could be comparable with other species of malaria such as *Plasmodium malariae*, which produces knobs but is not sequestered (17).

The study of *in vitro* binding with enzyme-treated erythrocytes provides some information on the nature of the surface molecules involved in sequestration. These molecules are associated at least in part with proteins of the infected erythrocyte membrane since binding is inhibited by treatment of infected blood with proteases. Proteases are only effective on erythrocytes containing parasites that have reached a certain degree of maturation. If protease treatment is applied earlier—i.e., at the ring stage, it has no effect on the results of the MCBA carried out once the parasites have reached a sufficient degree of maturation. This indicates either that the protease-sensitive molecules are of parasitic origin and are not yet exposed on the erythrocyte membrane at the ring stage or that, if a host component is involved, it becomes sensitive to protease treatment only after being first modified or exposed by parasite development. Increased binding of neuraminidase-treated erythrocytes infected with parasites from S^+ animals to melanoma cells could be related to a decrease in negative cell surface charge or to the exposure of a new carbohydrate residue involved in binding. The latter hypothesis assumes the presence of a lectin-like molecule on the surface of melanoma cells; such lectins have been described on various types of mammalian cells (18, 19). The increased reactivity of *P. falciparum*-infected squirrel monkey erythrocytes with certain lectins has previously been demonstrated (20), but the relationship between binding properties to lectins and to melanoma cells has not yet been found.

We have shown that antibody had an effect on the cytoadherence of infected erythrocytes *in vitro* and *in vivo*. The inhibition of *in vitro* binding of infected erythrocytes by immune serum indicates that antibody can interact with the structures of the erythrocyte that mediate binding. This interaction could be direct, through a mechanism of competitive inhibition, or indirect, through an antibody-mediated surface or cytoskeletal alteration of the membrane environment that inactivated the binding site. In a study performed in parallel, Hommel *et al.* (11) have shown by immunofluorescence that the surface of infected erythrocytes from S^+ animals reacted only with serum $31/S^+$ and that the surface of infected erythrocytes from S^- animals reacted only with serum $30/S^-$. The low inhibitory effect on binding of serum $30/S^-$, when compared with serum $31/S^+$, suggests a close linkage between the binding properties of the infected erythrocyte surface and the surface antigens detected on the infected erythrocyte by immunofluorescence.

In vivo, the sharp increase of circulating trophozoites/schizonts that occurs in an intact monkey within minutes after passive transfer of immune serum clearly indicates that the reversal of binding can occur *in vivo* as well as *in vitro*. The secondary decrease in parasitemia that is observed 24 hr later supports the view that an interaction of antibody with sequestration might represent the initial step of a chain of events that will eventually destroy the parasite. In the light of this inhibitory effect of immune serum on binding, it is of interest to note that the prevalence of cerebral malaria has been shown to be higher in children and in nonimmune individuals newly exposed to malaria (21). In some context, the "crisis forms" (i.e., deteriorating trophozoites and schizonts) that appear in the bloodstream of infected hosts at the onset of an effective immune response, just before the immune system finally resolves the infection (22, 23), might represent parasites whose binding to endothelial cells has been inhibited by circulating antibodies and that have subsequently been killed as a consequence of their presence in the peripheral bloodstream. Such observations could be related to the existence of antibody in the serum of immune individuals that would inhibit parasite sequestration, thus alleviating the clinical severity of the disease. The biochemical nature of the molecules present on the surface of the infected erythrocyte that mediate sequestration is not yet known, but it is conceivable that such molecules might constitute a potential immunogen.

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